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LECTIN AND RELATED PROTEINS
IN THE BARK OF *Sophora japonica* L.

Kei'ichi BABA

1990

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Introduction

Generally, trees are distinguished from herbs or grasses by their longer life, larger size and slower maturity. These characters indicate that the overground tissues of trees highly adapt themselves to surroundings with keeping their lives. At least, tree stem has a potential to cope with the seasonal changes of the climate. It is assumed that the adaptation to the climate is accompanied with functional changes of the tissues. The functional changes should be reflected on the changes in amount or species of proteins. The seasonally fluctuating proteins are expected to be a key to understand tree physiology.

In the earlier studies on the seasonal fluctuation of the substances in tree stem, the changes in the amount of total nitrogen, soluble nitrogen, and total protein were shown as that they are more in winter than in summer (for review, see Kramer and Kozlowski 1979). Only recently, however, few proteins were identified as the seasonally fluctuating proteins. One is a protein termed lectin, and the others are not characterized except for their molecular sizes. The former was shown in the bark of *Robinia pseudoacacia* and *Sambucus nigra* (Nsimba-Lubaki and Peumans 1986), the latter was shown in the bark of *Populus deltoides*, *Salix x smithiana* and *Acer saccharum* (Wetzel et al. 1989).

Lectin was found in the bark of many tree species (Krüpe and Ensgraber 1958; Hořejší et al. 1978; Gietl et al. 1979; Gietl and Ziegler 1980a, b; Hankins et al. 1988; Baba and Kuroda 1989), but its seasonal changes have only been investigated for the above two species. Moreover, it is still unknown whether the change in the amount of bark lectins is related to the differentiation of

tissue and/or development of bark. Some observations suggest that a certain annual phloem increment would be essential to the tree lives. Phloem production usually precedes the xylem production (for review, see Kozlowski 1971), and the annual increments of phloem occur in constant thickness within the species or individuals independently of the thickness of xylem increments (for review, see Kozlowski 1971), which vary by many conditions such as temperature, rainfall, defoliation; in the worst case, annual xylem production does not occur. It is important to reveal the relation between annual changes of the lectin and the tissue development of bark, in order to understand the role of lectin in the bark.

The other seasonally fluctuating proteins were found in the species other than those in which lectin fluctuates seasonally, as described above. Among these trees, only in *A. saccharum* two such polypeptides of 16 kDa and 24 kDa were found, but one polypeptide of 32 kDa was found in the others. There is also no information whether the other such proteins coexist with lectin in a species. It will provide basic information for understanding tree physiology to clarify how many proteins can be identified as seasonally fluctuating proteins, and how these changes occur in relation to season.

The present study was designed to clarify whether lectin shows a seasonal change even in the species other than those described above, *S. nigra* and *R. pseudoacacia*, how its seasonal change occurs within the bark in relation to the tissue development, and whether other proteins changing seasonally coexist in the same bark, by using *Sophora japonica* as a material. Lectins have been found in its seeds (Poretz et al.

1974; Allen and Johnson 1976; Timberlake et al. 1980)), leaves (Hankins et al. 1987) and branch bark (Hankins et al. 1988). To achieve these aims, immunochemical, immunofluorescence and immuno-gold electron microscopical techniques were adopted. These techniques allow to trace each certain protein.

Chapter 1

Lectin in the bark of *Sophora japonica*

Lectins are defined as non-immune (glyco)proteins which bind carbohydrate(s) to agglutinate cells and/or to precipitate glyco-conjugates (Goldstein et al. 1980). This definition implies that lectins are multivalent, that is, they possess at least two sugar binding sites. Because of their hemagglutinating activities and sugar binding properties, lectins are one of the very useful tools to determine the blood group, to detect sugar residue, to identify the kind of carbohydrate, and to isolate glyco-conjugates. Hence many lectins have been purified and characterized (for reviews, see Lis and Sharon 1986), and some of them are now utilized as such reagent. Lectins have been found in various tissues and organs of higher plants (for reviews, see Liener 1976; Etzler 1986).

Despite much information on purified plant lectins, their physiological roles in the plant body are still unclear. Within last 40 years, a number of hypotheses for the physiological roles of lectins were proposed (for reviews, see Etzler 1986); such as the involvements in defense mechanism of the plant, cell recognition, specific attractants for rhizobial symbiosis, cell wall elongation, carbohydrate catcher, and storage protein itself. Defense mechanism is mainly based on the interactions between several lectins and pathogens. For example, wheatgerm agglutinin, which has a specificity for chitin oligomers (Allen et al. 1973; Lotan and Sharon 1973), binds to chitin-containing cell walls of hyphal tips, septa and young spore of the fungi (Mirelman et al. 1975; Barkai-Golan et al. 1978), and inhibits

the growth and spore germination (Mirelman et al. 1975). Similar effects were also found in the lectins of potato (Garas and Kuc 1981), peanut and soybean (Barkai-Golan et al. 1978; Gibson et al. 1982). The symbiosis hypothesis is based upon the interaction between bacteria of the genus *Rhizobium* and leguminous plants. Importance of this idea was revived by Hamblin and Kent (1973), who reported the agglutination of *Rhizobium phaseoli* by seed extracts of *Phaseolus vulgaris*, a plant nodulated by this bacterium. They found that erythrocytes bound to the roots of this plant and could be agglutinated by the root extracts. Evidence on the possible involvement of lectins in cell wall elongation was reported by Roberts and Etzler (1984). They showed an association of the highest levels of the lectin (CRM) with the most rapidly growing internodes within 19-day-old plants of *Dolichos biflorus*. A significant level of this lectin appears to be associated with the cell wall (Etzler et al. 1984). "Carbohydrate catcher" means the aid of lectins in the transport of carbohydrates and their immobilization in the seeds. These hypotheses are based on the results with materials different from each other. Lectins have been found in the whole biological world, and even in a plant body, found in several tissues. Because the biochemical and physicochemical properties of lectins are different among the sources, the physiological role(s) should be elucidated distinctly in each source material.

Lectin in tree stems was first reported by Krüpe and Ensgraber (1958) as having hemagglutinating activity in crude extracts from *Laburnum alpinum*, *Cytisus praecox*, *Sophora japonica*, *Robinia pseudoacacia* and *Evonymus europaea*, and has since been confirmed in many other tree species. Gietl et al.

(1979) surveyed hemagglutinating activity in 53 dicotyledonous tree species. Some of these tree-stem lectins have been purified and characterized (Hořejší et al. 1978; Gietl et al. 1979; Gietl and Ziegler 1980a, b; Broekaert et al. 1984; Nsimba-Lubaki et al. 1986; Hankins et al. 1988; Baba and Kuroda 1989). In *Sambucus nigra* and *Robinia pseudoacacia*, the bark lectins were reported to fluctuate annually, their amounts increasing in autumn and decreasing in spring (Nsimba-Lubaki and Peumans 1986).

The bark lectin of *Sophora japonica* accounts for about 30% of the total soluble protein (Hankins et al. 1988, Baba and Kuroda 1989) and is the most abundant bark lectin thus far characterized. It is a tetramer constructed of about 30-35 kDa subunits, and has binding specificity for galactose derivatives (Hankins et al. 1988). The bark lectin differs from the lectins of seeds (Poretz et al. 1974) and leaves in *Sophora japonica* (Hankins et al. 1987), with regard to molecular size and subunit composition, indicating that the lectins are coded by distinct genes (Hankins et al. 1987, 1988). This bark lectin was demonstrated to be localized in the vacuole (Herman et al. 1988), but it is unknown which cells contain the lectin, whether the amounts fluctuate annually, and how they relate to the annual increments of the phloem tissues. It will be important to discuss the physiological role(s) of the lectins in the bark of tree stem. In Section 1.1, the studies were designed to resolve these problems.

The bark lectins are not only scientifically interesting, but also commercially valuable. As described in the first paragraph, some of the lectins are already utilized as reagents. If the bark lectin is capable of being used as those reagents,

the bark which is regarded as wasted parts in wood industry will have a commercial value. In Section 1.2, the lectin was purified from the trunk bark, which is more abundant than that of branch in a tree, and characterized. It was examined whether lectin is abundant also in the trunk bark as well as in the branch bark. All of the information on the bark lectin of *Sophora japonica* has so far been obtained from the branch bark (Krüpe and Ensgraber 1958; Hankins et al. 1988).

1.1 Localization and developmental changes in the bark lectin of *Sophora japonica*

There are few studies on the localization of bark lectin. Greenwood et al. (1986) demonstrated that the bark lectin of *Sambucus nigra* was stored in protein bodies, as observed with seed lectins of other species. Herman et al. (1988) demonstrated that lectin in bark cells of *Sophora japonica* was localized in vacuoles. However, the exact deposition of bark lectins in different cell types has yet to be determined. In the tree stem, cells which are living and may contain lectin are cells in cambial zone, sieve tubes, companion cells, and ray and axial parenchyma cells of xylem and phloem. Clarification of the exact localization of lectin in the bark is important for understanding its function in the tree.

Nsimba-Lubaki and Peumans (1986) reported that the bark lectins of *Sambucus nigra* and *Robinia pseudoacacia* have an annual rhythm, their amounts increasing in autumn and decreasing in spring. However, there is no information on other tree species and on the relation of this phenomenon to the development of

phloem tissue or to lectin localization. In temperate regions, the cambium of the stem, generally, produces phloem tissue from the beginning of spring to the middle of summer and adds it to the bark from the inside, the new tissue pushing the older outwards. This process is repeated year after year with the result that the older tissues are orderly arranged in annual rings from cambium to outer bark. There are many different patterns of the annual increment of phloem tissue and the formation of annual rings of inner bark (Esau 1969). In addition, because of the tensile stress induced by the enlargement of the stem in circumference, the parenchyma cells in the outer region of the inner bark enlarge their tangential diameter, and divide radially, thus increasing their number; the process is called "dilatation" (Esau 1969). To understand the function of lectin in the phloem tissue, it is important to clarify how biochemical changes are correlated with these histological changes.

The present study was designed to determine the exact lectin localization in different bark cell types of *Sophora japonica*, and to demonstrate how the lectin distribution and localization is correlated with the development of the phloem tissue. The pattern of annual growth and tissue accumulation of the bark were characterized histologically, and determined the changes in the bark lectin during the life of phloem parenchyma.

1.1.1 Materials and methods

Plant material, fixation and embedding.

An individual *Sophora japonica* growing on the campus of the Wood Research Institute, Kyoto University was used. The samples (approx. 1.1×0.5 cm) used for immunoblotting and enzyme-linked

immunosorbent assay (ELISA) were cut away at breast height from the stem (approx. 15 cm in diameter) of this tree by using a cutter knife. The samples were immediately frozen by liquid nitrogen, and stored at -80°C until use. To obtain samples for histological and immunocytochemical studies, tissue block (approx. 3.3×2 cm) was harvested from the same stem described above using a chisel and was cut into small pieces (approx. $0.5 \times 1 \times 2$ mm) in 4% paraformaldehyde, 0.2% glutaraldehyde, 0.5 mM CaCl_2 in 20 mM piperidine-N,N-bis(2-ethanesulfonic acid) (Pipes purchased from Dojin, Kumamoto, Japan) (pH 7.2). Some pieces were fixed in the solution described above at 4°C for 4 h. After washing with 20 mM 2-amino-(hydroxymethyl)-1,3-propanediol (Tris) buffer (pH 7.4) three times, they were dehydrated at 4°C using an ethanol series (50, 75, 90, 99 %) and embedded in hydrophilic methacrylate resin consisting of methyl methacrylate (Nacalai Tesque, Kyoto, Japan), glycol methacrylate (Nacalai Tesque) and Quetol 523M (Nisshin EM, Tokyo, Japan) (3:6:1, by vol.). The resin was cured either at 60°C and catalyzed by 0.05% 2,2,-azo-bis-iso-butyronitrile, or under ultraviolet irradiation at -10°C to -15°C and catalyzed by 1% benzoyl peroxide in gelatin capsules. Other pieces were fixed in 1.5% paraformaldehyde, 2.5% glutaraldehyde in Pipes buffer at 4°C for 8 h, washed with 20 mM sodium cacodylate buffer (pH 7.4) three times, and postfixed with 1% OsO_4 at 4°C overnight. After being washed with cacodylate buffer and dehydrated in an ethanol series (50, 70, 90, 95, 99%) and acetone, they were embedded in Spurr's low-viscosity resin (Polyscience, Warrington, Penn., USA).

Immunofluorescence and light microscopy.

Serial sections ($1\text{ }\mu\text{m}$ thick) were prepared from hydrophi-

lic-methacrylate-embedded samples with dry glass knives, mounted on glass slides with distilled water, and air-dried. All immunofluoro-staining procedures were carried out at 33°C in a moist chamber. The sections were incubated with 1% bovine serum albumin (BSA) in 10 mM sodium-phosphate-buffered saline (PBS) (pH 7.2) for 30 min, directly followed by incubation with fluorescein isothiocyanate (FITC)-labeled anti-*Sophora japonica* agglutinin (SJA) antibody (E. Y. Laboratories, San Mateo, Cal., USA), diluted 20-fold with 1% BSA in 10 mM PBS for 20 min. They were washed with 0.05% polyoxyethylene sorbitan monolaurate (Tween 20) in 10 mM PBS six times, 5 min each. Finally, sections were rinsed with distilled water, air-dried, and covered with 50% glycerol in PBS containing 1% *p*-phenylenediamine (Wako Pure Chemical Industries, Osaka, Japan), and were observed under a fluorescence microscope (BH-2 with BH-RFL; Olympus, Tokyo) conditioned for blue excitation and orange (O 530) barrier filtration.

The sections next to those used for immunofluoro-staining were stained with Coomassie brilliant blue G-250 (CBB) (Nacalai Tesque), and covered with 50% glycerol containing KI-I₂ (0.33% : 0.67%, w/v). Sections 1-2 µm thick were prepared from samples embedded in Spurr's resin, stained with Toluidine blue O (Chroma, Stuttgart, FRG) and mounted on glass slides. Both CBB- and Toluidine-blue-stained sections were observed under a conventional light microscope (BH-2; Olympus).

Immuno-gold electron microscopy.

Ultrathin sections were prepared from the Spurr's epoxy resin-embedded-samples and the hydrophilic methacrylate-embedded-samples (Methacrylate). The former was etched with 30% H₂O₂ and

then mounted on 150 mesh copper grids supported by folmvar film. The latter was directly mounted on the grids supported by folmvar film. Both H_2O_2 etched and the methacrylate-samples were treated with the immuno-gold detecting method. Sections on grids were blocked with 1% BSA in 10 mM PBS (pH 7.2) for 30 min, followed directly by incubation for 1 h with anti-SJA antibody (E.Y. Laboratories) diluted 20-fold with 0.1% BSA in 10 mM PBS. After washing with PBS six times, they were left at room temperature for 30 min with protein A-colloidal gold (Funakoshi Yakuhin, Tokyo) diluted 10-fold with 0.1% BSA in 10 mM PBS. They were then washed with 10 mM PBS twice, followed by distilled water six times. Lastly, they were air-dried and stained with uranyl acetate for 20 min, and observed under an electron microscope (H-700; Hitachi, Tokyo).

Two negative controls were prepared: one was the omission of anti-SJA antibody treatment from the immuno-gold procedure (control 1), and the other was the sections treated with preimmune serum instead of anti-SJA antibody (control 2). The gold-particle number per $1\ \mu\text{m}^2$ of cytoplasmic layer or intravacuolar area was determined. The values given are means with standard errors (SE) calculated by using 10 micrographs of approximate $50\ \mu\text{m}^2$ each of immuno-gold, control-1, or control-2 preparation.

Conventional electron microscopy.

Ultrathin sections were prepared from Spurr's resin-embedded sample. They were mounted on 150 mesh copper grids supported by folmvar film, and stained with uranyl acetate and lead citrate.

Immunoblotting.

Bark, harvested in June and February and stored in frozen condition, was cut tangentially with a cryostat into 50- μ m-thick sections from the inside to the outside of the bark. The resulting sections were collected in lots of ten and placed into Eppendorf tubes, to which 500 μ l of 125 mM Tris buffer containing 8 M urea and 5% sodium dodecylsulfate (SDS) was added. The sections were sonicated with a Sonifier Cell Disrupter 200 (Branson, Danbury, Conn., USA) and centrifuged at 10,000 $\cdot g$ for 15 min. The protein contents of the supernatants were determined using the bicinchoninic acid (BCA) Protein Assay Reagent (Pierce, Rockford, Ill., USA). Then, β -mercaptoethanol was added to 5% concentration and the aliquots were boiled for 5 min, followed by centrifugation at 10,000 $\cdot g$ for 15 min. The supernatants were charged as equal protein amounts on 12% polyacrylamide gel (9.6 \cdot 0.1 cm) and subjected to electrophoresis using the discontinuous buffer system of Laemmli (1970). The proteins in the gel were then stained with Coomassie brilliant blue R250 (Nacalai Tesque) or electrically transferred to a nitrocellulose sheet (45- μ m pores) by the method of Towbin (1979). After washing with 0.05% Tween 20, 500 mM NaCl in 20 mM Tris-HCl (pH 7.5) (TTBS) for 10 min, the transblotted nitrocellulose sheet was blocked with 5% skimmed milk (Morinaga Seika, Tokyo) in TTBS at 4°C overnight. It was then left at room temperature for 1 h with anti-SJA antibody diluted 1000-fold with 0.1% BSA in TTBS. After this was washed with 5% skimmed milk in TTBS six times, 5 min each, and was then left at room temperature for 1 h with alkaline-phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Organon Teknika Corp.-Cappel Product, West Chester, Penn.,

USA) diluted 3000-fold with 0.1% BSA in TTBS. Subsequenting this it was washed again six times with TTBS, and then with 150 mM barbital sodium buffer (pH 9.6) for 10 min. Finally, the proteins were made visible with nitroblue-tetrazolium (Nacalai Tesque) and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma Chemical Co., St. Louis, Mo., USA) (Blake et al. 1984).

Enzyme linked immunosorbent assay (ELISA).

To each specimen, which consisted of either the total inner bark harvested monthly or the tangential slices of June and February samples described in the previous section, 0.1 M sodium carbonate buffer (pH 9.7) was added it 1 μ l/mg of fresh weight of the specimens. The specimens were sonicated and centrifuged at 10,000·g. Each supernatant was diluted linearly in ten stages from 0- to 10,000-fold and 50 μ l of each was applied to 96-well EIA plates (Coster, Boston, Mass., USA) and left at 4°C overnight. The wells of the plates were then washed with 0.05% Tween 20 in 0.1 M phosphate buffered saline (TPBS) three times, and blocked with 1% BSA in PBS for 30 min. After blocking, anti-SJA antibody diluted 1000-fold was applied, and the preparations incubated for 1 h. After this they were washed with TPBS three times and peroxidase-conjugated anti-rabbit antibody (Bio-Rad, Richmond, Va., USA), diluted 3000-fold, was applied, followed by another incubation for 1 h. The wells were then washed again three times with TPBS; color was developed with o-phenylenediamine (Wako Pure Chemical Industries, Osaka) and absorbance was measured at 492 nm.

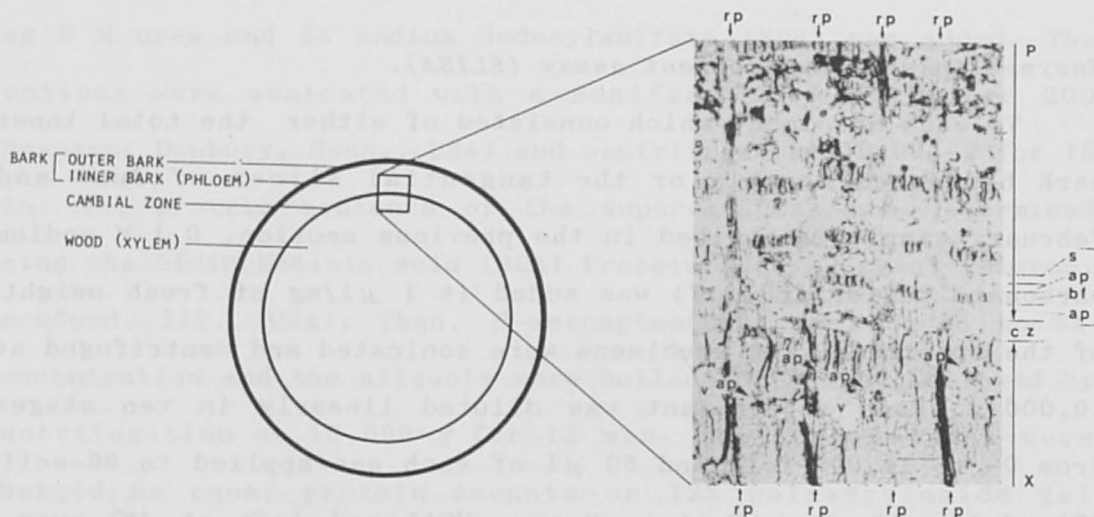


Fig. 1.1 Light microscope image of a cross section of the cambium from a stem of *Sophora japonica*.

The section was prepared from a sample harvested in December from the portion illustrated as a rectangle in the diagram on the left (diameter=15 cm approx.), embedded in Spurr's resin and stained with Toluidine blue O. CZ, cambial zone; X, xylem; P, phloem; rp, ray parenchyma; ap, axial parenchyma; bf, bast fibers; s, sieve tubes v, vessel. Bar=100 μ m.

1.1.2 Results

Lectin localization in the stem.

As illustrated in Figure 1.1, the stem of *Sophora japonica* consists of wood (xylem), cambial zone and bark. The bark consists of an inner bark (phloem), a living tissue, and an outer bark, a dead tissue. A cross section of the stem, corresponding to the rectangular area in the diagrammatic illustration, shows that ray parenchyma (rp) aligned radially from cambium (C) to xylem (X) and phloem (P). In the phloem, axial elements are aligned as follows. Bands of axial parenchyma (ap), bast fibers (bf), axial parenchyma (ap) and sieve tubes (s) are repeated from the cambium to the outer bark. In the xylem of this field of view, most of the axial elements consists of fibers, with some axial parenchyma (ap) and vessels (v) were also present. Completely differentiated bast fibers, xylem fibers in the xylem and vessels consist only of cell walls.

Lectin was localized by immunofluorescence microscopy. A cross section near the cambium is shown in Figure 1.2 at somewhat greater magnification than the field shown in Figure 1.1. Lectin was detected as green fluorescence. The cell walls of bast fibers and xylem cells appeared yellow because of autofluorescence of lignin. Lectin was located mainly in both axial and ray phloem parenchyma. No green fluorescence was observed in the cells of cambial zone, the bast fibers, vessels and xylem fibers. The parenchyma cells in the xylem also appeared to contain lectin, but in low intensity of the fluorescence. Even when the area between bast-fiber bands was observed under higher magnification (Fig. 1.3), sieve tubes (s) and companion cells (arrowheads) were found to contain no lectin (Fig. 1.3A) although protein was

observed by staining with Coomassie brilliant blue and KI/I₂ (Fig. 1.3B). These localization patterns were not changed in any other season (data not shown).

Lectin was found not only in the phloem but also in the xylem. Xylem lectin of *Sophora japonica* has not been reported so far. In order to determine whether the lectin in the phloem and in the xylem are identical, they were analyzed by immunoblotting (Fig. 1.4). While the phloem lectin showed apparent molecular sizes of 32, 33.5 and 35 kDa, which were consistent with findings in a previous report (Baba and Kuroda 1989), the xylem lectin showed a different molecular size of 40 kDa. Comparing the phloem lectin with the xylem lectin on SDS-PAGE, the former was dominant, but the latter was minor in the proteins. These results suggest that the xylem lectin might be a different species from the phloem lectin. Further it have been demonstrated that their intracellular localization. Figures 1.5 and 1.6 represent respectively electron micrographs of the phloem and the xylem ray parenchyma cells. They were quite different from each other with regard to cell content (Figs. 1.5A and 1.6A). The phloem cell had many vacuoles of various sizes of 1-8 μm in diameter, containing many electron-dense clumps. Furthermore, the phloem cell was characterized by the presence of many oil droplets and a few small starch grains, approx. 1.5 μm in diameter. On the other hand, the xylem cell was distinguished by vacuoles of similar size, approx. 3 μm in diameter, containing no electron-dense clumps and many large starch grains, approx. 3 μm in diameter. Lectin localization in these two different types of ray parenchyma cells was demonstrated by using immuno-gold staining method. In the phloem cell, gold particles were found only in the

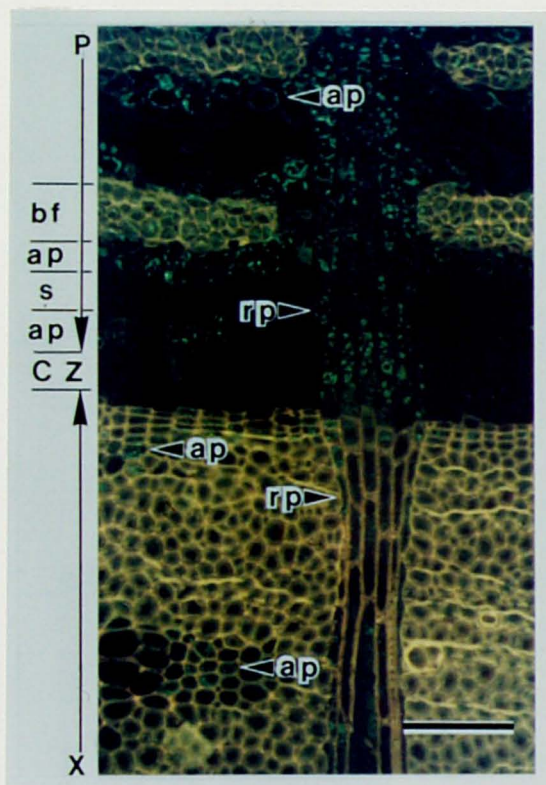


Fig. 1.2 Immunofluorescence localization of lectin in the stem of *Sophora japonica*.

Lectin is recognized as green fluorescence; it can be detected in ray parenchyma (rp) and axial parenchyma (ap) of both the phloem and the xylem. CZ, cambial zone; X, xylem; P, phloem; bf, bast fibers; s, sieve tubes. The sample was harvested in December. Bars= 100 μ m.

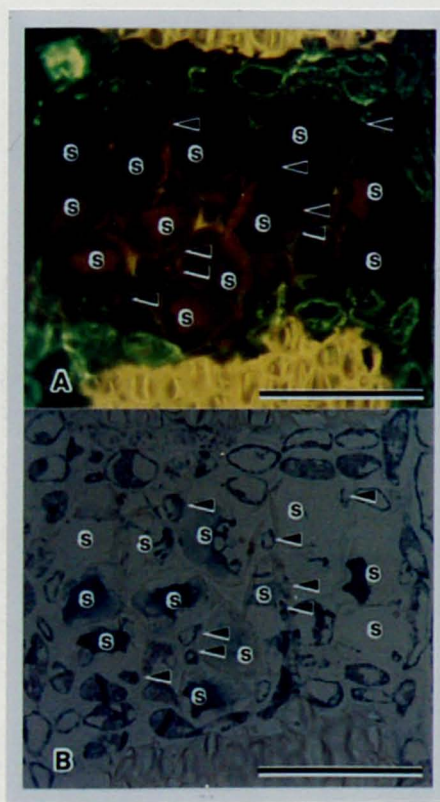


Fig. 1.3 A, B Immunofluorescence and Coomassie-blue-staining images of sieve tubes and companion cells in the bark of *Sophora japonica*.

A: Immunofluorescence localization of lectin; it can not be detected in the sieve tubes (s) and companion cells (arrowheads). B: Protein and starch stained with CBB and KI/I₂ respectively. The sample was harvested in December. Bars=100 μ m.

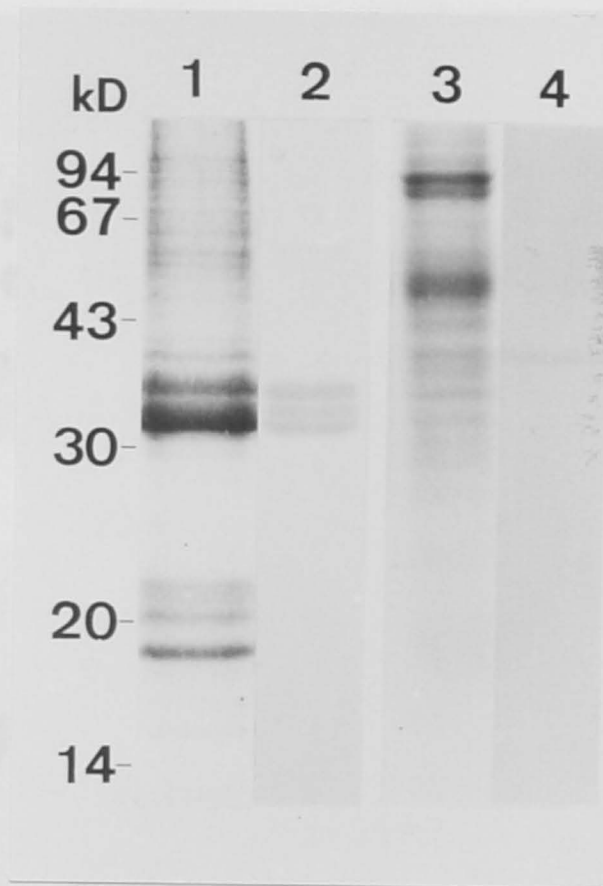


Fig. 1.4 SDS-polyacrylamide gel electrophoresis and immunoblotting of the proteins extracted from the phloem and xylem of *Sophora japonica*.

Lanes 1 and 2 are proteins from crude extracts of the phloem. Lanes 3 and 4 are proteins from crude extracts of the xylem. Lanes 1 and 3, CBB staining. Lanes 2 and 4, immunoblotting detected with anti-SJA antibodies. Numbers on the left represent apparent molecular sizes.

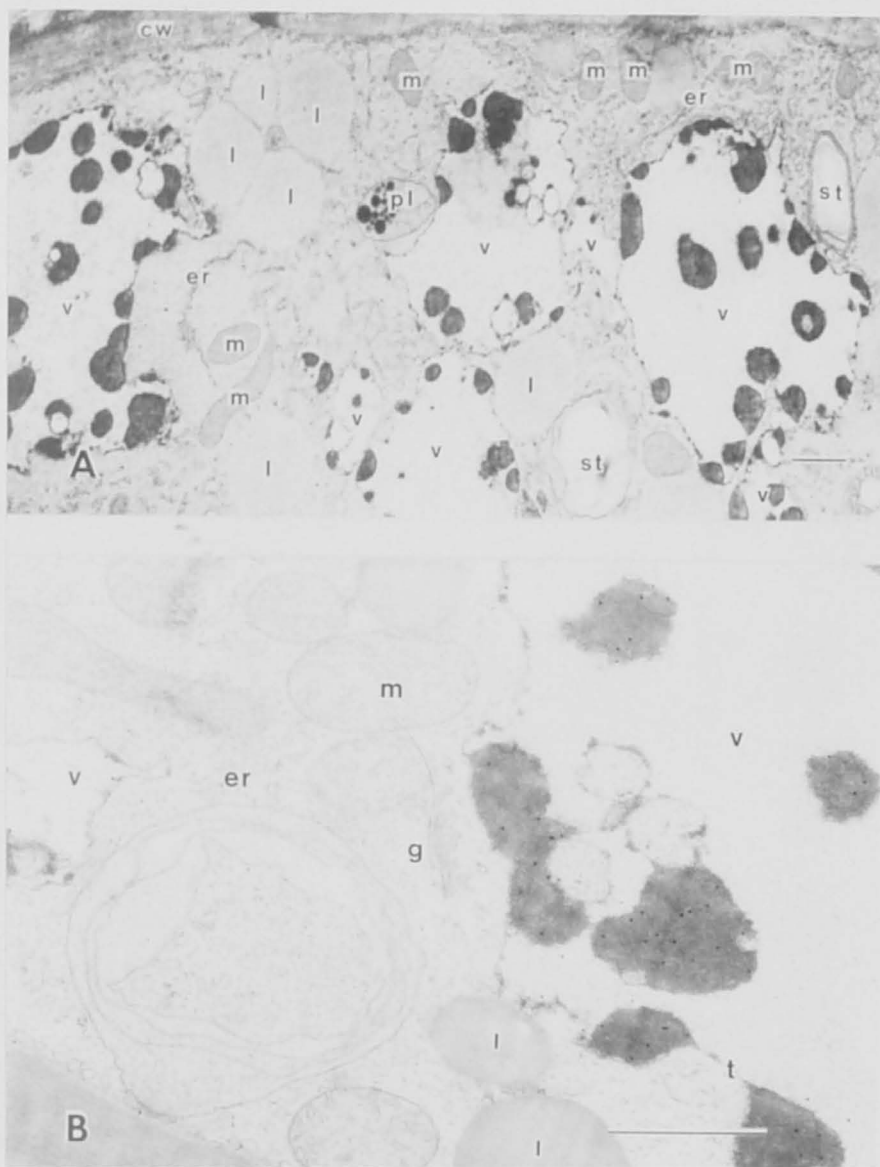


Fig. 1.5 A, B Immuno-gold electron micrographs of a phloem ray parenchyma cell of *Sophora japonica*.

A: An electron micrograph by conventional method.

B: Immuno-gold staining of lectin. Gold particles were observed on electron-dense cluster in vacuole.

cw, cell wall; er, endoplasmic reticulum; g, Golgi apparatus; l, lipid or oil droplet; m, mitochondrion; pl, plastid; st, starch; v, vacuole. The sample was harvested in December or September. Bars=1 μ m.

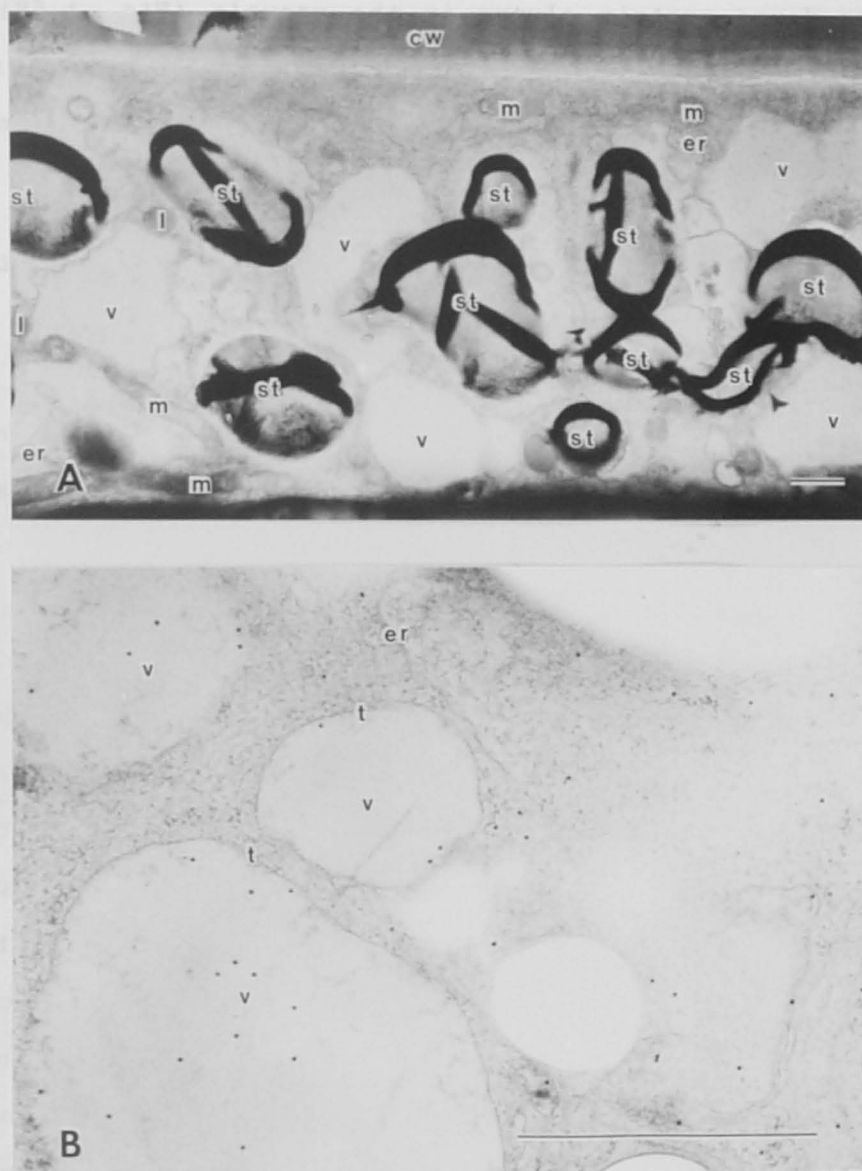


Fig. 1.6 A, B Immuno-gold electron micrographs of a xylem ray parenchyma cell.

A: An electron micrograph by conventional method.

B: Immuno-gold staining of lectin. Gold particles are observed within vacuole and ER.

cw, cell wall; er, endoplasmic reticulum; l, lipid or oil droplet; m, mitochondrion; pl, plastid; st, starch; v, vacuole.

The sample was harvested in December. Bars=1 μ m.

electron-dense protein clumps in the vacuole (Fig. 1.5B), while in the xylem cell, the gold particles were also distributed within the vacuoles, except for many gold particles observed on the endoplasmic reticulum in the cytoplasm (Fig. 1.6B).

Histological characterization of the annual growth of the bark tissue of Sophora japonica.

Annual growth of the bark (the secondary phloem) of *Sophora japonica* was studied histologically. Transverse sections near the cambium of the trunk, collected from March to July, are shown in Figure 1.7. In March, the cambium was still dormant. In April, the cell number and radial diameter of the cells in the cambium increased, indicating that the latter had begun to divide. From May onwards the new tissue, derived from the cambium, began to differentiate, and sieve tubes in the tissue of the previous year began to undergo destruction. By June the first bast fiber band of the year was differentiated, and sieve tubes in the old tissue were now completely destroyed. Afterwards, bands of axial parenchyma, sieve tubes, axial parenchyma and bast fibers were repeated, and three or four fiber bands were produced until the end of summer. Ray parenchyma cells were produced continuously.

The view of a whole bark sample is shown in Figure 1.8. Distortions of the alignment (arrowheads) occurred on each ray parenchyma at almost equal spaces (small arrows). Obviously in the July panel of Figure 1.7, the earliest interval between fiber bands of the year was wider and there were more sieve tubes present in it than in the other later intervals. Hence these distortions, arising because of the destruction of sieve tubes, indicate the early tissue of each year, and the tissue between one distortion and the next indicates that produced in a year.

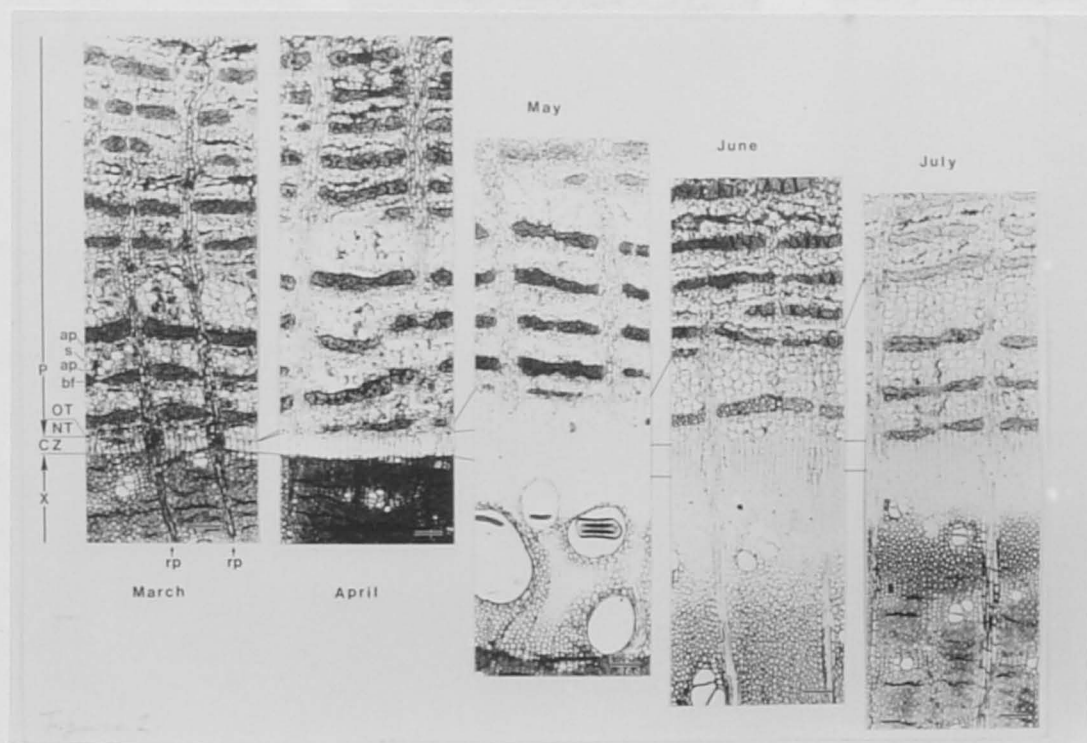


Fig. 1.7 Annual growth of secondary phloem of *Sophora japonica*. These transverse sections were prepared from the samples embedded in Spurr's epoxy resin and stained with Toluidine blue O. CZ, cambial zone; P, phloem; X, xylem; NT, new tissue of the current year; OT, old tissue; bf, bast fibers; s, sieve tubes; ap, axial parenchyma; rp, ray parenchyma. Bars=100 μ m.



Fig. 1.8 A transverse section through a bark sample from a main stem of *Sophora japonica*.

This bark sample was harvested in June. The bottom end is cambium and the top end is the outer bark. Distortions occurred on the align of the ray parenchyma (arrowheads). The small arrows on the left indicate the position of the distortions which are the borders of the tissue age. The cells positioned by a large arrow increased their tangential diameter, indicating the initiation of dilatation. The numbers of 500- μ m sections on the right correspond to the sample numbers on immunoblots and ELISA data of Fig. 1.10 . Bar=1 mm.

Therefore, this bark (Fig. 1.8) was determined to contain the living tissues formed in the past seven years at least. The tangential diameter of the cells was enlarged at the border of five-year-old and six-year-old tissue (large arrow in Fig. 1.8), indicating that dilatation had been initiated, and the cells positioned exterior to that point were more randomly arranged than those which were interior.

Annual changes in the amount of bark lectin.

The amount of lectin was measured throughout a year on a monthly basis by ELISA (Fig. 1.9). Lectin levels were at a peak in December, and a high level, about 80% of the peak, was maintained until March. The amount of lectin rapidly decreased to about 35% from March to May. It appeared to increase slightly from May to August, with some fluctuation, and then increased rapidly from September to November. The average amount of lectin in spring and summer, from May to August, amounted to about 50% of that in winter, from November to March.

In order to determine whether the changes described above occurred uniformly within the inner bark, bark samples were stripped off in June and February, dissected from the inside to the outside into 500 μm sections, and the proteins in each section were immunoblotted (Fig. 1.10A). The section numbers in Figure 1.10 correspond to those in Figure 1.8. While the amounts did not differ greatly among the February sections (Fig. 1.10A, "F"), the amount of lectin in section 1 of June was markedly less than that in the other sections (Fig. 1.10A, "J").

The lectin content, on a fresh-weight basis, of each section was measured by ELISA (Fig. 1.10B), and the relative amounts are shown as the heights of the columns. A notable finding in the

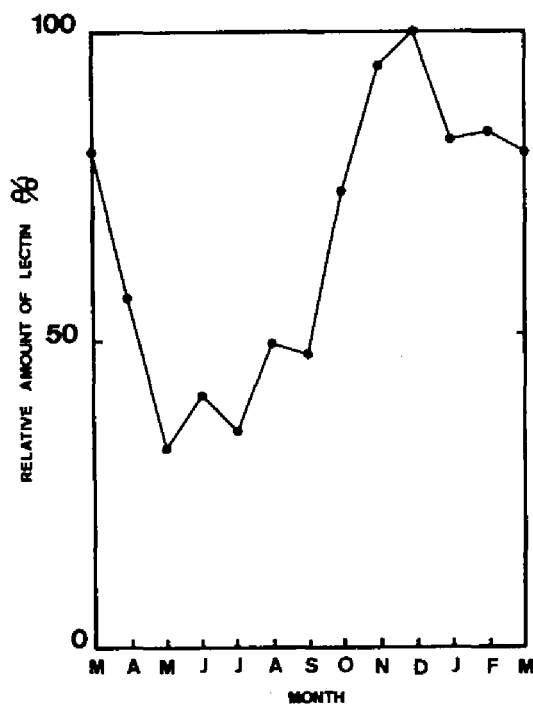


Fig. 1.9 Annual changes in the amount of bark lectin in *Sophora japonica*.

The amounts were determined by ELISA, and are shown as percentages of the value in December, at which time lectin is most abundant.

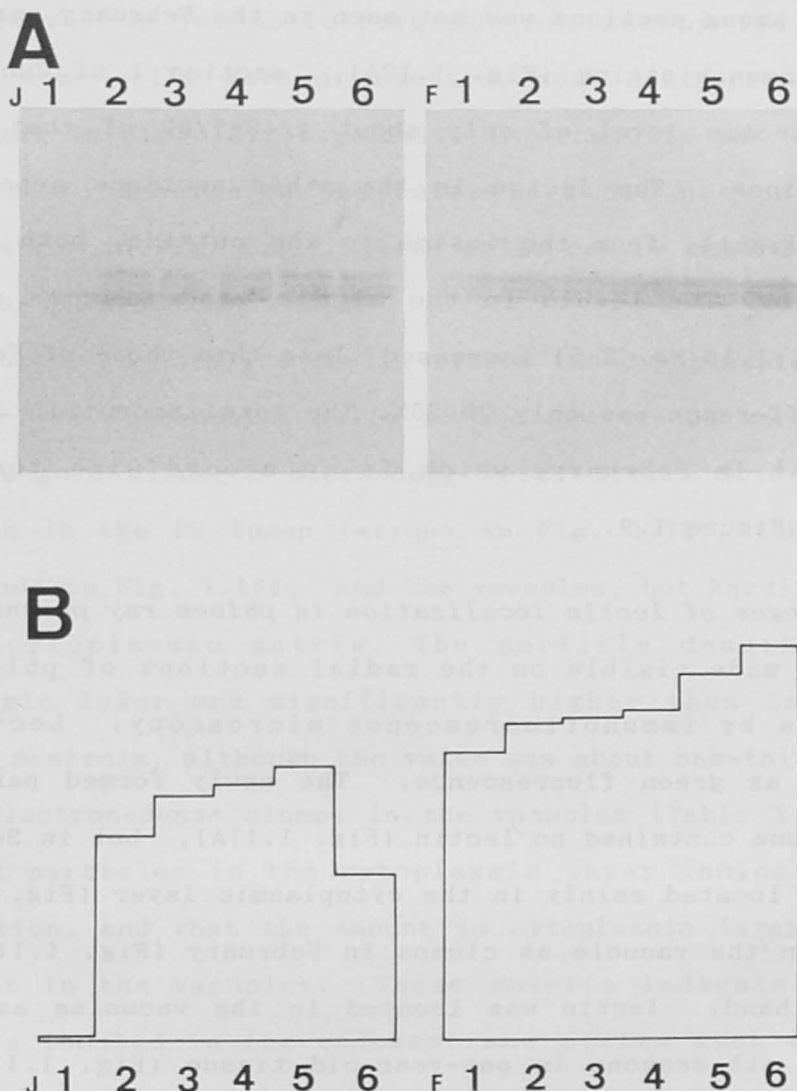


Fig. 1.10 A, B Immunoblotting and ELISA of sectioned bark of *Sophora japonica* in June and February.

The sample numbers correspond to the tissues of the same numbers in Fig. 1.8.

A: Immunoblotting of sectioned bark.

B: Relative lectin amount of each section determined by ELISA.

J = June; F = February.

June sample, which had never been observed by immunoblotting, was that the lectin level of the outermost section (Fig. 1.10B, "J", No. 6) was markedly lower than that of the middle sections. This difference among sections was not seen in the February sample. As shown by immunoblotting (Fig. 1.10A), section 1 of June had a very low lectin level of only about 1/40-1/60 of that in the other sections. The lectin in the other sections appeared to increase slightly from the inside to the outside, both in June and February. The levels in the middle-aged tissues of June (Figs. 1.8, 1.10 No. 2-5) increased less than those of February, but the difference was only 20-25%. The total amount in June was 50% of that in February, which is in accord with the ELISA results in Figure 1.9.

Annual changes of lectin localization in phloem ray parenchyma.

Lectin made visible on the radial sections of phloem ray parenchyma by immunofluorescence microscopy. Lectin was recognized as green fluorescence. The newly formed parenchyma cells in June contained no lectin (Fig. 1.11A), but in September lectin was located mainly in the cytoplasmic layer (Fig. 1.11C), and only in the vacuole as clumps in February (Fig. 1.11E). On the other hand, lectin was located in the vacuoles as clumps throughout all seasons in one-year-old tissue (Fig. 1.11B,D,F). The cytoplasmic layer of some cells in one-year-old tissue contained lectin in September (arrowheads in Fig. 1.11D), although the intensity was much less than in new tissue (Fig. 1.11C). The tissues older than those in September also contained lectin in the cytoplasmic layer (data not shown), but at even lower levels.

As shown in Figure 1.10B, the amount of lectin in the

outermost section (No. 6) in June was less than that in the middle sections. In order to investigate the changes in localization patterns, the outermost region (Fig. 1.8, No. 6) was also observed by immunofluorescence microscopy (Fig. 1.12). Although some cells gave a reaction, most contained no lectin (arrowheads in Fig. 12). Especially important is the fact that the cells which bordered the outer bark did not contain any lectin (arrow in Fig. 1.12). These cells can be regarded as cork cambium or phellodermal cells.

In order to localize lectin within the cytoplasmic layer in September, ray parenchyma cells in the new tissue were observed by immuno-gold electron microscopy (Fig. 1.13). Gold particles were seen in the ER lumen (arrows in Fig. 1.13), the vesicles (arrowheads in Fig. 1.13), and the vacuoles, but hardly observed in the cytoplasmic matrix. The particle density in the cytoplasmic layer was significantly higher than in the two negative controls, although the value was about one-third of that in the electron-dense clumps in the vacuoles (Table 1.1). Hence the gold particles in the cytoplasmic layer indicate lectin localization, and that the amount in cytoplasmic layer is lower than that in the vacuoles. These results indicate that the lectin is located in the endomembrane system such as ER and vesicles in autumn and is evidently transported into the vacuole before winter.

The deposition route of the bark lectin.

Lectin was localized in the ER lumen in autumn, and in the vacuole in winter. The route from the ER to the vacuole was studied by using immuno- and conventional electron microscopy. On

Fig. 1.11 A-F Immunofluorescence micrographs of radial sections of the phloem ray parenchyma of *Sophora japonica*. Lectin is detected as green fluorescence. A, B, harvested in June; C, D, harvested in September; E, F, harvested in February. A, C, E, tissue formed in the current year; B, D, F tissue formed in the previous year. Arrowheads=cells containing lectin in both vacuolar clump and cytoplasmic layer. Bars=100 μ m.

Fig. 1.12 Immunofluorescence micrograph of the radial section of the outermost region of living bark of *Sophora japonica* harvested in June.

Lectin is detected as green fluorescence. I, inner bark (living bark); O, outer bark; arrowheads, cells containing no lectin; arrow, cells bordering on outer bark. Bar=100 μ m.

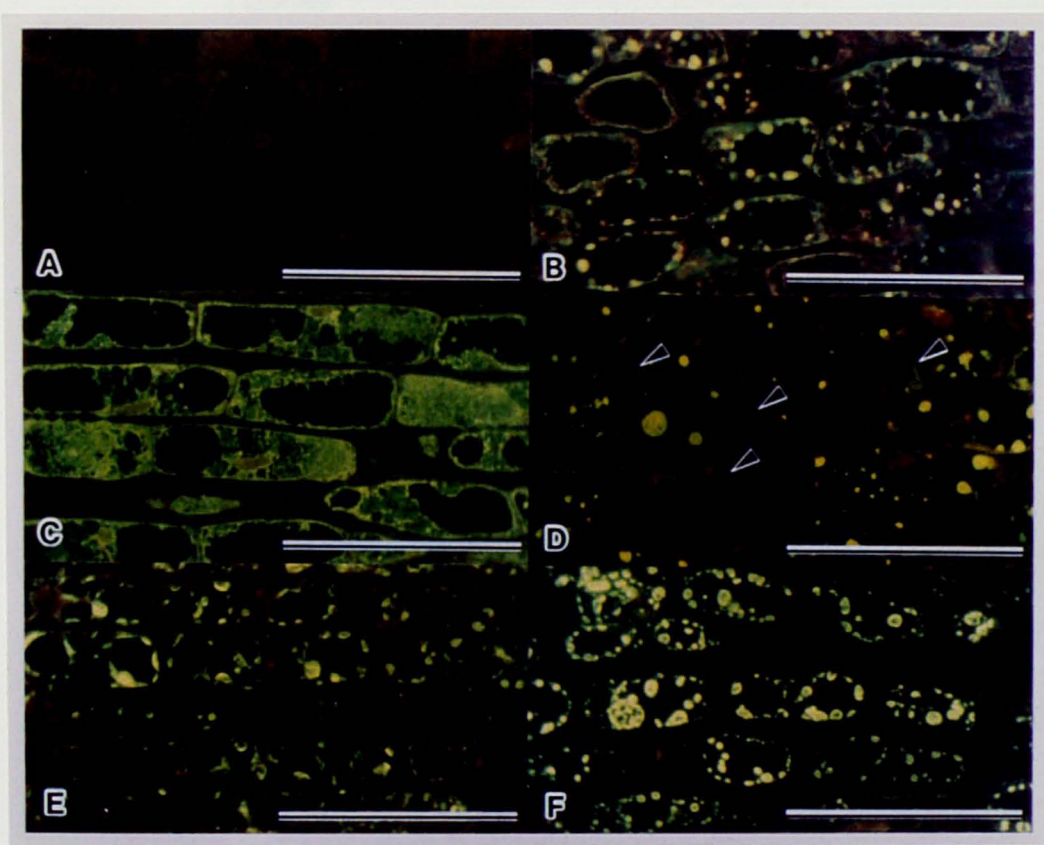


Fig. 1.11 A-F

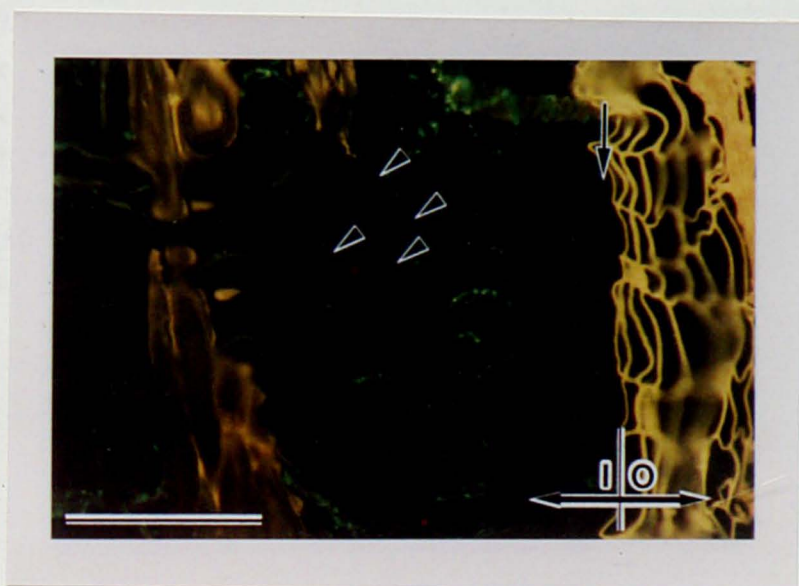


Fig. 1.12

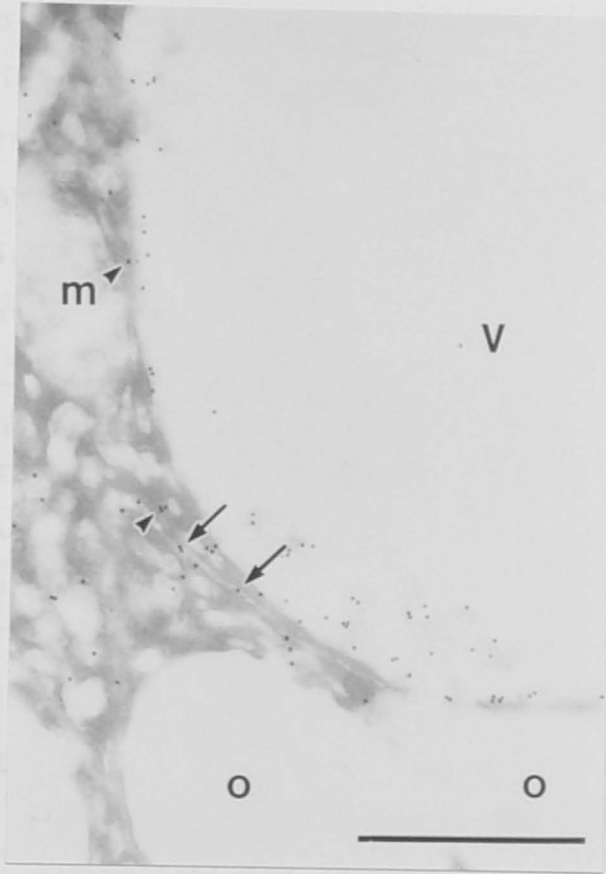


Fig. 1.13 Immuno-gold electron micrograph of the section of phloem ray parenchyma of *Sophora japonica* in September.

Gold particles are seen on materials in vacuole, ER (arrows), and vesicles (arrowheads). v, vacuole; m, mitochondrion; o, probably trace of oil droplet extracted during the dehydration because it is not fixed by osmium. tetroxide Bar = 1 μ m.

Table 1.1 Densities of gold particles on the phloem ray parenchyma cells in new bark tissue of *Sophora japonica* embedded in hydrophilic methacrylate.

	No. gold particles μm^{-2} (mean \pm S)
Control 1	0.21 \pm 0.06
Control 2	0.046 \pm 0.018
Immuno-gold	
V	7.2 \pm 2.2
C	2.5 \pm 0.5

Control 1 = treatment with only gold-labeled protein A, no antibody treatment. Control 2 = Treatment with preimmune rabbit serum instead of the antibody. Immuno-gold = immuno-gold electron microscopy; V = the electron-dense clumps in the vacuole; C = cytoplasmic layer. Each value was calculated by using ten micrographs of approx. $50 \mu\text{m}^2$.

the sections prepared from H₂O₂-etched-sample harvested in September, gold particles, indicating lectin localization, were observed only in the electron-dense clumps of the vacuole (Fig. 1.14). The gold particles were not observed on any other organelle. However, Golgi apparatus was often seen at just back of the electron-dense clumps (Fig. 1.14). Moreover, the tonoplast between the Golgi cisternae and the clump formed several small invagination (arrows in Fig. 1.14B), and the electron-dense material was observed between the invagination and the clumps (arrowhead in Fig. 1.14B). On the sections prepared from methacrylate sample gold particles were observed in the lumen of the Golgi apparatus (arrows in Fig. 1.15) and its vesicles (arrowheads in Fig. 1.15), and of course in the vacuoles. On the other hand, in the observation by the conventional electron microscopy, electron-dense materials were observed in the lumen of the ER-like endomembrane system (Fig. 1.16A), and moreover, it was sometimes observed that the ER-like endomembrane containing the electron-dense materials appeared to fuse to the tonoplast directly (Fig. 1.16B).

1.1.3 Discussion

The bark lectin of *Sophora japonica* changed in the course of the year relative to the season, with the amount in spring and summer from May to August being about 50% of that in winter from November to March (Fig. 1.9). However, this annual change of lectin content did not occur uniformly throughout the bark. Whereas the amount in winter was similar from the inside to the outside of the bark, the inner- and outermost regions contained less lectin than the middle region of the bark in summer (Fig. 1.10B). These changes are mainly explained by proposing that, in

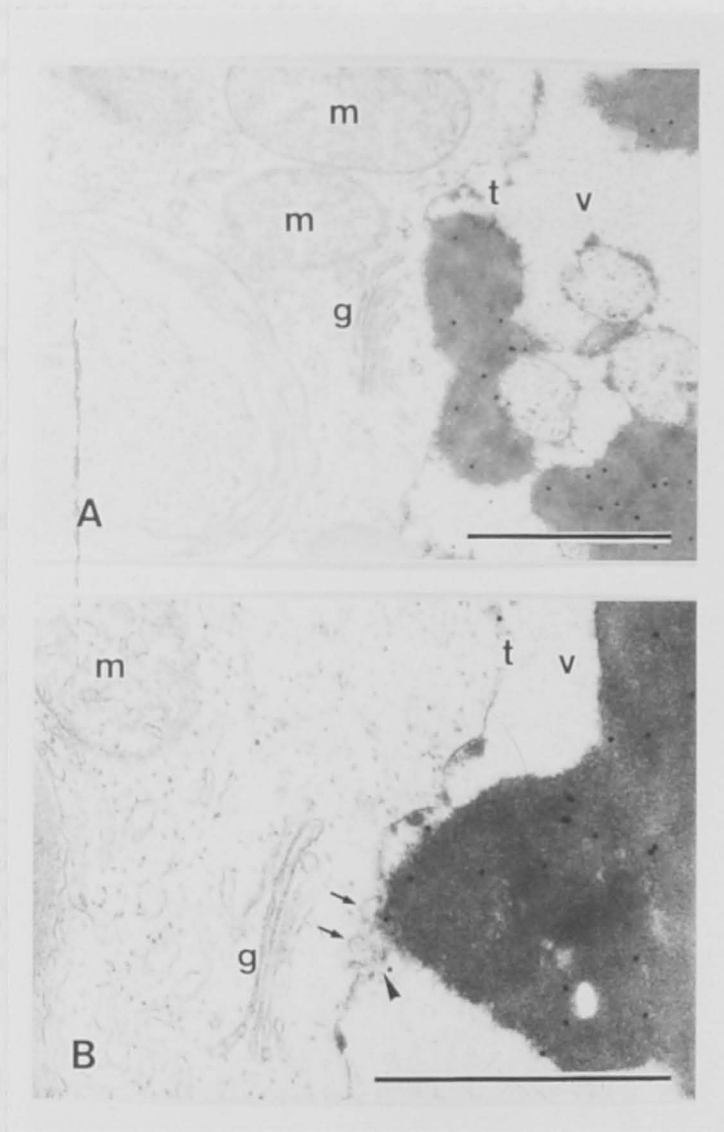


Fig. 1.14 A, B Immuno-gold electron micrographs of the sections of the phloem parenchyma cell of *Sophora japonica* etched by H_2O_2 after embedding into Spurr's epoxy resin.

A: Gold particles are observed on the electron-dense materials in the vacuole, and the Golgi apparatus was observed at just back of the electron-dense clump.

B: The tonoplast between the electron-dense clumps and the Golgi apparatus forms invagination (arrows), and electron-dense materials were observed between the invagination and the clumps (arrowhead) g, Golgi apparatus; m, mitochondrion; t, tonoplast; v, vacuole. The sample was harvested in September. Bars = 1 μm .

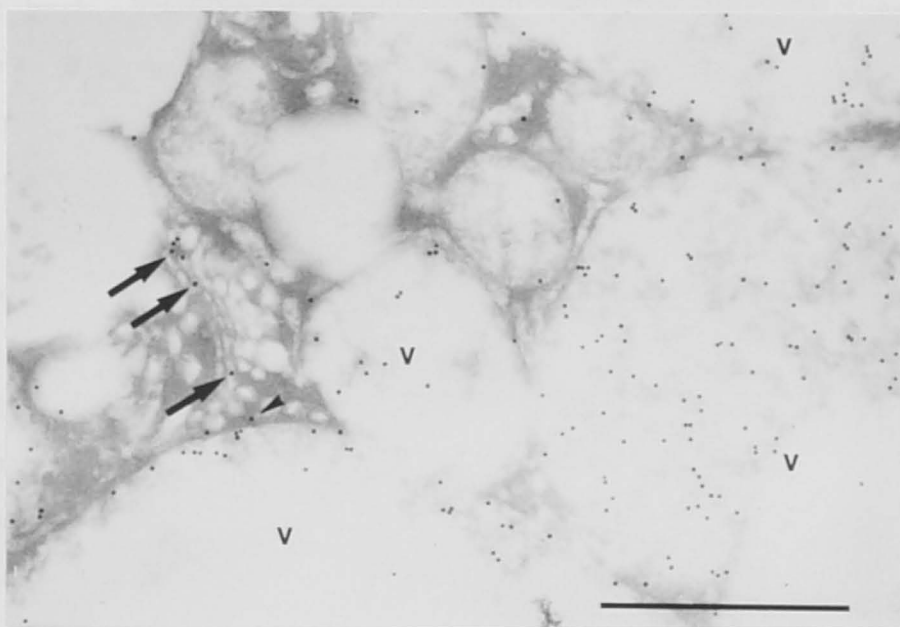


Fig. 1.15 Immuno-gold electron micrographs of the sections of the phloem parenchyma cell of *Sophora japonica* embedded in hydrophilic methacrylate resin. The sample was harvested in September.

Gold particles are seen on the materials in vacuole, Golgi apparatus (arrows) and vesicles (arrowhead). v, vacuole. Bars = 1 μ m.

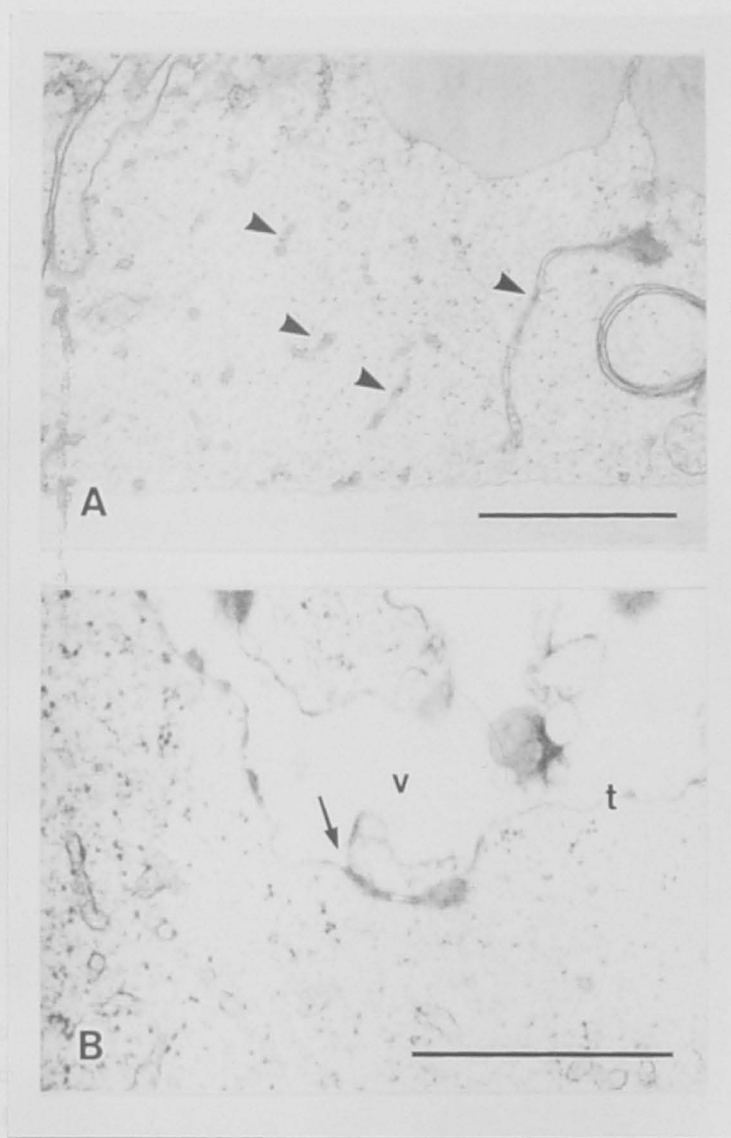


Fig. 1.16 A, B Conventional electron micrographs of the phloem ray parenchyma cells of *Sophora japonica*.

A: The ER-like endomembrane was filled with the electron-dense materials (arrowheads).

B: The ER-like endomembrane containing the electron-dense materials appeared to fuse to tonoplast (arrow). t, tonoplast; v, vacuole.

The sample was harvested in September.

Bars = 1 μ m.

spring and summer, new tissue, which contains no lectin, is added to the bark from the inside while the cells in the outermost part degrade lectin, so that the total amount of lectin decreases; in autumn the new tissue synthesizes lectin while the outermost tissue, in which lectin has been exhausted, dies and becomes outer bark, so that the total lectin amount in the inner bark increases. The present immunocytochemical data support this interpretation. In spring and summer, the new tissue contained no lectin (Fig. 1.11C), and lectin completely disappeared in some cells of the outermost region and in all the cells bordering on the outer bark (Fig. 1.12). In autumn lectin was located in the lumen of the ER and vesicles in the cells of the new tissue (Fig. 1.13). The latter observation is consistent with the findings for several seed storage proteins in developing cotyledons (Baumgartner et al. 1980; Craig and Goodchild 1984; Herman and Shannon 1984; Greenwood and Chrispeels 1985). Biochemical studies have demonstrated that these proteins are synthesized on ER-attached ribosomes and sorted into the ER lumen (Bollini and Chrispeels 1979; Bollini et al. 1982; Chrispeels and Bollini 1982; Chrispeels et al. 1982; Hurkman and Beevers 1982; Faye and Chrispeels 1987), and it may be assumed that the bark lectin is also synthesized on ER-attached ribosomes. Because in the middle-aged tissue the cytoplasmic layer of the cells also contained lectin in autumn (Fig. 1.11D), these tissues appear to retain the potential for lectin synthesis, although it is not as high as in the new tissue. This may explain why the amount of lectin increases slightly from the inside to the outside of the bark. The lectin in the middle part in June comprises only 75% of that in February. This may be because tissues of every age mobilize

small amounts of lectin, or that samples could not be harvested from exactly the same position.

Concerning with the deposition route of the lectin, the results suggest two route models. The results on the immuno-gold electron microscopy (Figs. 1.14, 15) suggest that the bark lectin may pass through Golgi apparatus. This deposition route is also demonstrated in the other storage proteins in seeds of dicotyledons (Bollini and Chrispeels 1978; Chrispeels et al. 1982; Chrispeels 1983). In contrast, the results on the conventional electron microscopy (Fig. 1.16) suggest that ER may be directly involved in the secretion of electron-dense materials to vacuole. It is possible that only some type(s) of lectin molecules or some subunit(s) may be transported through the Golgi and the others directly from the ER to the vacuole. The bark lectin of *Sophora japonica* is tetrameric molecules constructed with three or more types of subunits (Hankins et al. 1988; Baba and Kuroda 1989), and is mixture of the five molecular species which are able to be separated with ion-exchange chromatography (Hankins et al. 1988). The two-route models, that some type is transported via Golgi apparatus and the other type bypassing the Golgi within the certain protein species, was also proposed on the secretion of α -amylase in the barley germination (Akazawa and Hara-Nishimura 1985).

Lectin was located in both axial and ray parenchyma cells of the phloem of *Sophora japonica*. The other living cells, i.e., cambium, sieve tubes and companion cells, hardly contained any lectin. Therefore, like the annual changes this lectin localization indicates that bark lectin has no direct relation to secondary growth in thickness or to nutrient transport, but is a

storage or storage-related protein, as was proposed by Greenwood et al. (1986) and Nsimba-Lubaki et al. (1986).

It was further proposed that the lectin is utilized primarily by the phloem-parenchyma cells themselves rather than being transported to the leaves in the next year. The nutrients required by leaves are supplied through the tracheids and-or vessels of the xylem. Of the carbohydrates stored in the stem, starch in the contact cells (the members of ray parenchyma in the xylem which provide the connection between the living ray system and the vessels) is mobilized prior to that in the other ray cells (Sauter 1972). In contrast, the bark lectin decreased mostly in the outermost region of the bark, and it appear unlikely that the resulting degradation products can be transported to the xylem, across the cambium. On the other hand, dilatation (see above) in the outer part of the inner bark occurs during the active period of secondary growth (Esau 1969, pp. 183-197), and cork-cambium differentiates and is active in the same region (Esau 1977). The bark lectin may be assumed to be consumed *in situ* in these two processes. Because the amount of bark lectin decreased rapidly from March to May, it may be further assumed that the lectin is involved in these processes mainly at their beginning, when the supply of assimilates from other parts of the tree is not yet sufficient. There remains a possibility that some bark lectin is degraded from spring to summer and the resulting amino acids are transported to the cambium through the ray parenchyma and utilized in cambial growth. The observed loss of lectin in middle-aged tissues in summer, although small, may be the consequence of such a utilization.

1.2 Purification and characterization of the bark lectin of *Sophora japonica*

Some of well-characterized lectins, mainly obtained from the seeds of Leguminosae, are now available as reagents to identify carbohydrates, isolate glycoconjugates and so on. Lectins were also found in the barks of trunks or branches described as in introduction of this chapter. If such lectins are able to be isolated by simple and economical procedures, inner barks and branches, which are the waste parts of trees, could become more valuable for the wood industry.

The seed lectin of *Sophora japonica* is well-known as SJA (*Sophora japonica* agglutinin) (Poretz et al., 1974; Allen and Johnson, 1976; Timberlake et al., 1980), and have been already utilized as a reagent. Recently, lectin was purified from the branch bark of *Sophora japonica* (Hankins et al., 1988). But to some extent, the branch bark is anatomically different from the trunk bark. The trunk bark looks promising as another important lectin resource. Therefore, lectin was purified from the trunk bark and partially characterized and compared it with SJA as a reference.

1.2.1 Materials and methods

Purification of the lectin.

Bark with cambial zone (26 g fresh weight) of a *Sophora japonica* was harvested in June 1985 and squeezed by using a vise. The sap thus collected was centrifuged at 1000·g for 10 min (Fraction 1: 6 ml of the supernatant). Proteins in Fraction 1 were precipitated by adding ethanol to 50% concentration at -20°C. The resulting precipitate was collected by centrifugation

(8000·g, 30 min). The precipitate was dissolved in phosphate-buffered saline (PBS, pH 7.8) and centrifuged (Fraction 2: 15 ml of the supernatant). Fraction 2 was affinity-chromatographed on an acid-treated agarose column (1.5cm Ø x 30 cm, flow rate: 18 ml/hr) by the method of Allen and Johnson (1976). After the sample was applied to the column, the bed was washed with 20 mM Tris buffered saline (TBS, pH 8.7) and collected every 6 ml. Then the adsorbed fraction was eluted with 0.2M galactose in TBS. This effluent was collected every 3 ml. After dialysis against PBS, they were tested for their agglutinating activities. The active fractions were combined to give Fraction 3 (14.5 ml). Finally, they were dialyzed against distilled water and lyophilized.

Protein assay.

Protein contents were determined by a modified Lowry method (Bensadoun and Weinstein, 1976) to avoid interference by phenolics. Proteins in samples were collected by precipitation with sodium deoxycholate and trichloroacetic acid followed by the reaction with Folin-Ciocalteu reagent. A calibration curve was obtained by employing bovine serum albumin as a standard.

Hemagglutination assay.

The strength of hemagglutinating activity of each sample was described as its threshold dilution folds showing the activity. Samples were diluted in two-fold increments and pipetted into 96 well titer plates each 50 µl. After 50 µl of type B erythrocyte suspension (2%) in PBS (pH 7.8) and 25 µl of the PBS were added to each well, they were incubated at 27°C for 1h. Their agglutination images were judged under a microscope after the mixtures were agitated.

Polyacrylamide gel electrophoresis (PAGE)

Purified lectin was dissolved in 10% sucrose and electrophoresed under pH 4.3 or pH 8.9 according to the method of Reisfeld et al. (1962) or Davis (1964).

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Purified lectin was dissolved in 125 mM Tris-HCl (pH 6.8) containing 8 M urea, 4% SDS and 5% β -mercaptoethanol. Then it was electrophoresed using discontinuous buffer system of Laemmli (1970).

Gel filtration chromatography.

A Sephacryl S-300 (Pharmacia Fine Chemicals) column (0.95 cm \varnothing x 100 cm, void volume: 32 ml) was used. Lyophilized lectin (2.5 mg in 200 μ l of PBS(pH 7.2)) was applied to the column and eluted with PBS (pH 7.2) at 9.0 ml/hr. The effluent was monitored at 280 nm. The column was calibrated by using Combithek (calibration proteins kit; Boehringer Mannheim GmbH. Biochemica).

1.2.2 Results and discussion

The trunk bark lectin of *S. japonica* was purified by the following procedures: extraction of crude juice, ethanol precipitation and affinity chromatography (Fig 1.17). Volume, protein amounts and agglutinating activity were measured at every stages (Table 1.2). The yield was 27% of total protein in the extracts. Comparing with the other tree species, in which lectin ranged from 5 to 10% of the total proteins (Gietl et al., 1979; Broekaert et al., 1984; Nsimba-Lubaki et al., 1986), the trunk bark of *Sophora japonica* abounds in lectin. This result is accord

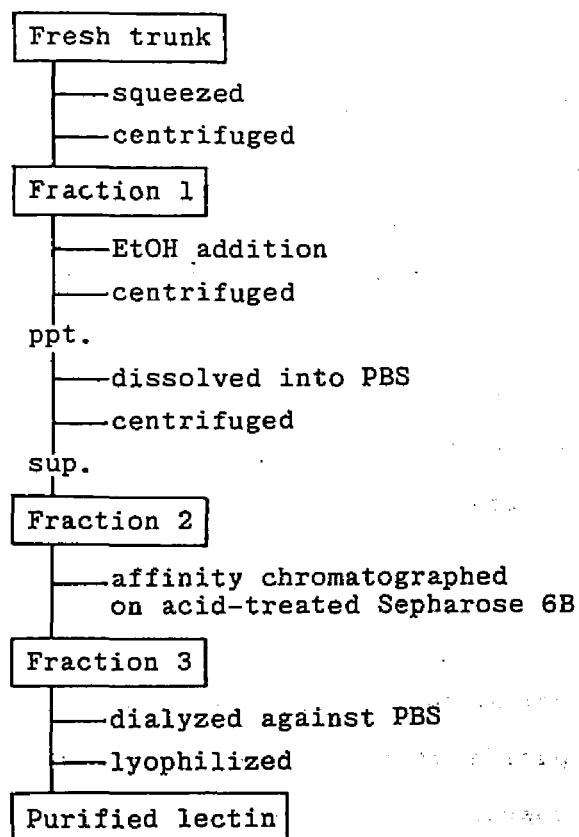


Fig. 1.17 Purification scheme of *Sophora japonica* trunk bark lectin.

Table 1.2 Purification of the trunk bark lectin of *Sophora japonica*.

Fraction	Vol. (ml)	Act. (titer)	Conc. (mg/ml)	Act. (titer x ml)	Total Protein (mg)	Specific Activity
1	6.0	2560	3.54	15360	19.4	790
2	15.0	960	0.92	14400	13.8	1040
3	14.5	626	0.37	9090	5.3	1710

Fraction 1, 2 and 3 are crude juice, solution obtained by ethanol precipitation and by affinity chromatography, respectively. Volume (Vol.), hemagglutinating activity (Act.) and protein concentration (Conc.) are experimental data. Total activity (Total Act.), protein content (Total Protein) and Specific Activity are calculated data.

Table 1.3 Hemagglutinating activities of the fractions during purification of the trunk bark lectin of *Sophora japonica*.

Fraction	Blood Types		
	A	B	O
1	5120	5120	5120
2	640	640	640
3	640	640	640

Activities for type A, B, and O were obtained by the same way as described in the text.

with the report on the branch bark of this species (Hankins et al., 1988). They reported that it contains 30% of lectin in its total protein.

Figure 1.18 shows the elution profiles of protein and agglutinating activity on the affinity chromatogram (the final step of purification). Agglutinating activity was detected only in the fraction of second peak of A_{280} : after galactose was added. This figure indicates that the bark lectin was able to be purified by the method used for the seed lectin of the same species, essentially (Allen and Johnson, 1976). When the crude juice fraction was directly applied to the affinity column, the agglutination activity was detected in both peaks before and after galactose addition. In other words, the lectin in the crude extracts did not fully adsorb to the bed. When the ethanol precipitated fraction was added, the lectin was completely adsorbed to the bed. Instead of ethanol precipitation, gel filtration (Sephadex G-25) led to the same result (the data are not shown). These findings suggested that the crude juice of a *Sophora japonica* bark contains a factor which partially impedes the adsorption of the lectin to the affinity bed. This factor is probably a low molecular material, because it is removed by a gel filtration (data not shown). The impeding factor is possibly related to the physiological roles in the bark.

The bark lectin was analyzed by gel filtration and polyacrylamide gel electrophoresis (PAGE). The trunk bark lectin showed a single peak on a gel filtration chromatogram, and its molecular weight was 135000 ± 5000 (Fig. 1.19). This result, which was in accordance with ultracentrifugation data (Baba 1987), suggests that this lectin might have a single molecular weight

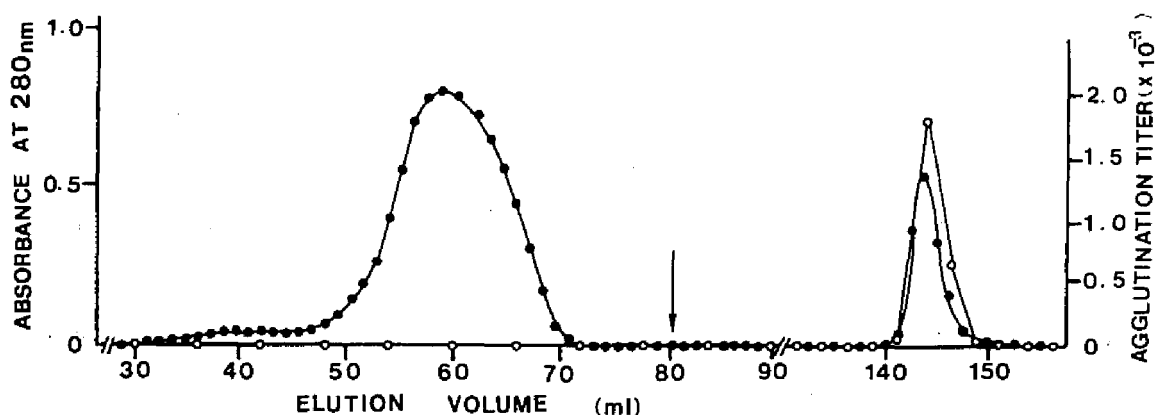


Fig. 1.18 Elution profile of *Sophora japonica* trunk bark lectin on acid-treated Sepharose 6B.

At the point indicated by an arrow, 0.2M galactose was added to the elution solvent (20 mM TBS; pH 8.7). Closed circles show absorbance at 280 nm (A_{280}). Open circles show hemagglutinating activity. No activity and absorbance were found in 0-30 and 90-130 ml fractions, so that they were omitted.

and be similar to that of the seed lectin reported as 132,800 (Poretz et al., 1974). On the other hand, when analyzed by using native PAGE, the bark lectin showed a different migration pattern from that of the seed lectin both under acidic and basic condition (Fig. 1.20 A and B). The bark lectin showed multiple bands under both of the conditions and their patterns were different from those of the seed lectin. These findings suggest that the bark and seed lectins are different in their molecular species. This suggestion was supported by the data of SDS-PAGE and observation of hemagglutination specificity. Where the seed lectin showed a main band of 33 kDa and minor bands of 34.5 and 36 kDa, the bark lectin showed a main band of 32 kDa and minor bands of 33.5 and 35 kDa (Fig. 1.20 C). The trunk bark lectin showed a different specificity for blood types from the seed lectin. The seed lectin was specific for types A and B (Krupe and Braun, 1952; Poretz et al., 1974), whereas the trunk lectin equally agglutinated types A, B and O (Table 1.3).

Comparing with the seed lectin, the bark lectin of *S. japonica* had a similar molecular weight, different charge variants, different molecular weights of subunits, a broader specificity for the human blood types. These results demonstrate that the bark lectin is different from the seed lectin. The bark lectin showed multiple bands on PAGE under both acidic and basic conditions, while the seed lectin showed three under basic and a single under acidic condition. The peculiar band patterns of the seed lectin is ascribed to the conformational changes but not to the constructive variation of the subunits (Timberlake et al., 1980). On the other hand, the reason why the bark lectin shows the multiple band patterns was unknown. Hankins et al.

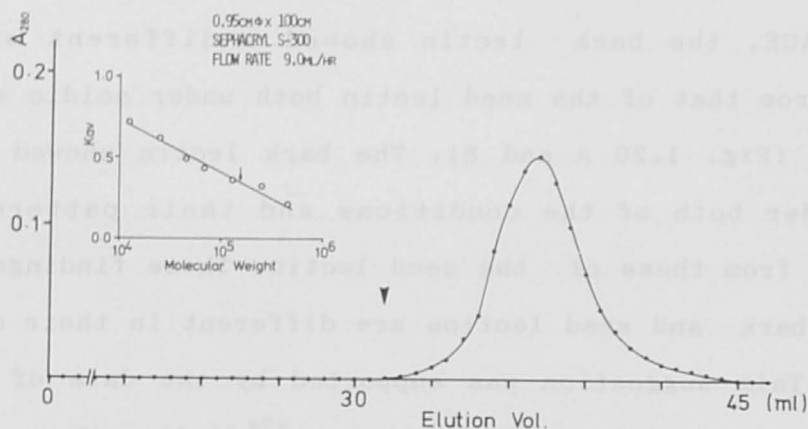


Fig. 1.19 Gel filtration chromatogram of the purified trunk bark lectin of *Sophora japonica* on Sephacryl S-300.

Void volume of this column (0.95 cm Ø x 100 cm) was found to be 32 ml (arrowhead) by using blue-dextran solution. The calibration of this column was shown. The arrow indicates the molecular weight of the bark lectin.

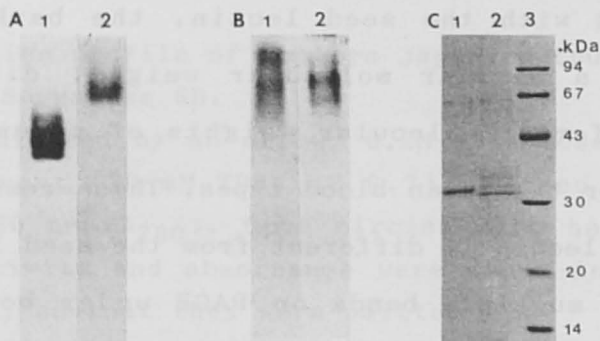


Fig. 1.20 Analysis of trunk bark and seed lectins of *Sophora japonica* by PAGE.

A: pH 4.0, native. B: pH 8.9, native. C: SDS-PAGE. Lane 1 is purified seed lectin. Lane 2 is purified trunk bark lectin. Lane 3 is Mr marker.

(1988) have just reported five lectins, which are different in their charge, from the lectin of branch bark in *S. japonica*. In the present study, multiple PAGE pattern might be caused by such charge variant shown in their report.

The trunk bark lectin has one dominant subunit of 32kDa and two minor subunits of 33.5 kDa and 35 kDa shown by using SDS-PAGE, whereas branch bark lectin was reported to have two major subunits of 30 kDa and 33 kDa and a trace of 30.1 kDa (Hankins et al., 1988). Although trunk and branch are apparently similar, being different in their age, they have some anatomical differences. One of the most different characters between their barks is: Branch, or young, living bark consists of cortex, which originates from ground meristem, and phloem, which originates from cambium, while trunk living bark consists of only the latter. Especially, the stem of *Sophora japonica* keeps cortex for a long time, approximately 20-30 mm in diameter. Such anatomical difference between the branch and trunk may cause the difference of their subunits. In this species, different tissues contain different lectins from one another (Hankins et al., 1987; 1988). On the other hand, the trunk bark lectin may vary with geographical population still also remains unsolved. Poretz et al. (1974) discussed that the seed lectin of *Sophora japonica* may vary with geographical population.

It was shown herein that the trunk bark lectin of *Sophora japonica* is able to be purified by a simple method: ethanol precipitation and affinity chromatography. The affinity bed is also prepared easily. Because the trunk bark and seed lectins are different in their molecular species, the trunk bark lectin may become valuable as another carbohydrate-binding reagent.

Chapter 2

Other proteins fluctuating seasonally in the bark of

Sophora japonica

As described in Chapter 1, lectin is a dominant protein in the bark of *Sophora japonica* and its amount changes seasonally: it increases in autumn, the high level in amount is kept during winter, and it decreases in spring. This result is accord with that on the other species, *Sambucus nigra* and *Robinia pseudoacacia*. However it is still unknown whether the other seasonal fluctuating protein coexists with lectin in the same bark.

The present study was designed to identify other seasonal fluctuating proteins within the bark of *Sophora japonica*, and to determine their annual changes quantitatively. The proteins showing the similar pattern to lectin are targeted. In order to investigate such protein, the migration pattern in winter was compared with that in summer by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). After antibodies were raised against what are found in those PAGE, their seasonal changes were measured by enzyme-linked immunosorbent assay (ELISA).

2.1 Material and methods

Plant material.

The same tree of *S. japonica* described in Chapter 1 was used. Specimens were harvested monthly from the main stem (about 15 cm in diameter) and immediately put into liquid nitrogen. They were stored at -80°C until use.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

The bark samples harvested in June and in December were used. The procedure of the electrophoresis was conducted as described in chapter 1.

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE).

Each specimen was cut into about 1 mm³ cubes and put into an Eppendorf tube with lysis buffer containing 8 M urea, 2% Nonidet P-40 (Sigma Chemical Co., St.Louis, Mo., USA), 5% β -mercaptoethanol, 0.5% SDS. After squashed with glass-rod, they were sonicated by using a Sonifier Cell Disruptor 200 (Branson, Danbury, Conn, USA). The resulting suspension was centrifuged at 10,000 \cdot g for 15 min. The supernatant was recovered as the extracts.

The extracts were analyzed by 2D-PAGE according to the method of O'Ferell (1975). The isoelectric focusing gel contained 2% ampholine (one part of pH 3.5-10 and one part of pH 5-7) (LKB, Sweden). For second dimension, 12% polyacrylamide gel was used.

Antiserum.

The polypeptide spots in the 2D-PAGE gels stained with CBB were cut off and electrically eluted from the gel by using an electro-eluter (Nippon Eido, Tokyo, Japan). After dialyzed against distilled water, the eluted polypeptides were lyophilized. They were dissolved into PBS, and emulsified with Freund's complete adjuvant. The resulting emulsions were injected under the skin of rabbits. After following four times injection

using incomplete adjuvant instead of the complete adjuvant week by week, the whole blood of each rabbit was collected a week after the last injection. After leaving at room temperature at least 30 min, they were centrifuged at 500·g for 20 min, and antiserum against each polypeptide was obtained.

Enzyme linked immunosorbent assay (ELISA).

To each sample, harvested monthly, 0.1 M carbonate buffer (pH 9.7) was added in 1 μ l/mg fresh weight. They were sonicated and centrifuged. Each supernatant was diluted in ten stages linearly from 0- to 10,000-folds and 50 μ l of each was used to assay. The procedure was carried out as described in chapter 1.

2.2 Results and discussion

Figure 2.1 shows the major changes occurred in the bark proteins of *Sophora japonica* between summer (June) and winter (December), analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The most obvious change was that the polypeptide band of 18 kDa in molecular size was present only in December. The three major bands between 32-35 kDa which were seen in both lanes were subunits of lectin as described in chapter 1. In order to search other changes in relation to season, the bark proteins in June and in December were analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), and shown in Fig 2.2. The 18 kDa band, found on SDS-PAGE, was not a single polypeptide, but observed as a group of 3 or more polypeptide spots spreading in pI = 7.0-8.3. Other than the group of 18 kDa, three polypeptide spots were found as the obvious changes appearing in December and disappearing in June: 22 kDa, pI 9.1, 20 kDa, pI 6.3, and 21 kDa, pI 6.2. Among these polypeptides, two

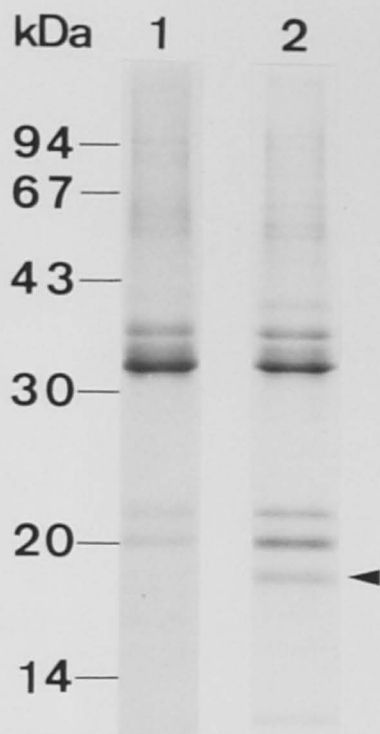


Fig. 2.1 SDS-polyacrylamide gel electrophoresis of the bark proteins of *Sophora japonica*.

Lane 1, harvested in June; Lane 2, harvested in December.

Arrowhead indicates the band of 18 kDa observed only in December.

polypeptides were purified and antibodies against them were obtained; one was the polypeptides of 22 kDa (arrow 1 in Fig. 2.2B), and the other was the group of 18 kDa (arrow 2 in Fig. 2.2B), because they gave larger spots on the gel, indicating that their amounts are larger than the others. The former was named WP22 (winter polypeptide of 22 kDa), the latter WP18.

By using these antibodies the annual changes of their amounts were measured on monthly basis (Fig. 2.3, 2.4). The contents per fresh weight of each month was shown as relative value of the most abundant month, March. Both of them were abundant in winter and less in summer, but the changing profiles were somewhat different from each other. WP22 decreased linearly from March until July when it disappeared completely, and increased linearly until the next March (Fig. 2.2). WP18 decreased from March to June as well as WP22, however, it did not completely disappear even in summer, and was retained about 20% of the peak. Then in autumn, the amount of WP18 was kept about 50% with a little fluctuation, and increased with a large fluctuation from the end of autumn. By the observation on 2-D PAGE, WP 18 was apparently absent in summer, but the results on ELISA showed that it was present even in summer. Perhaps the amount in summer was too little to be detected by the staining with Coomassie brilliant blue.

Although the profiles of WP22 and WP18 were dissimilar especially in their increasing period, both of them were abundant in winter and less in summer. And the difference of the amounts between winter and summer of them were greater than that of the lectin. The amount changes of WP22 and WP18 were distinguishable by PAGE level, whereas the change in the lectin was not found

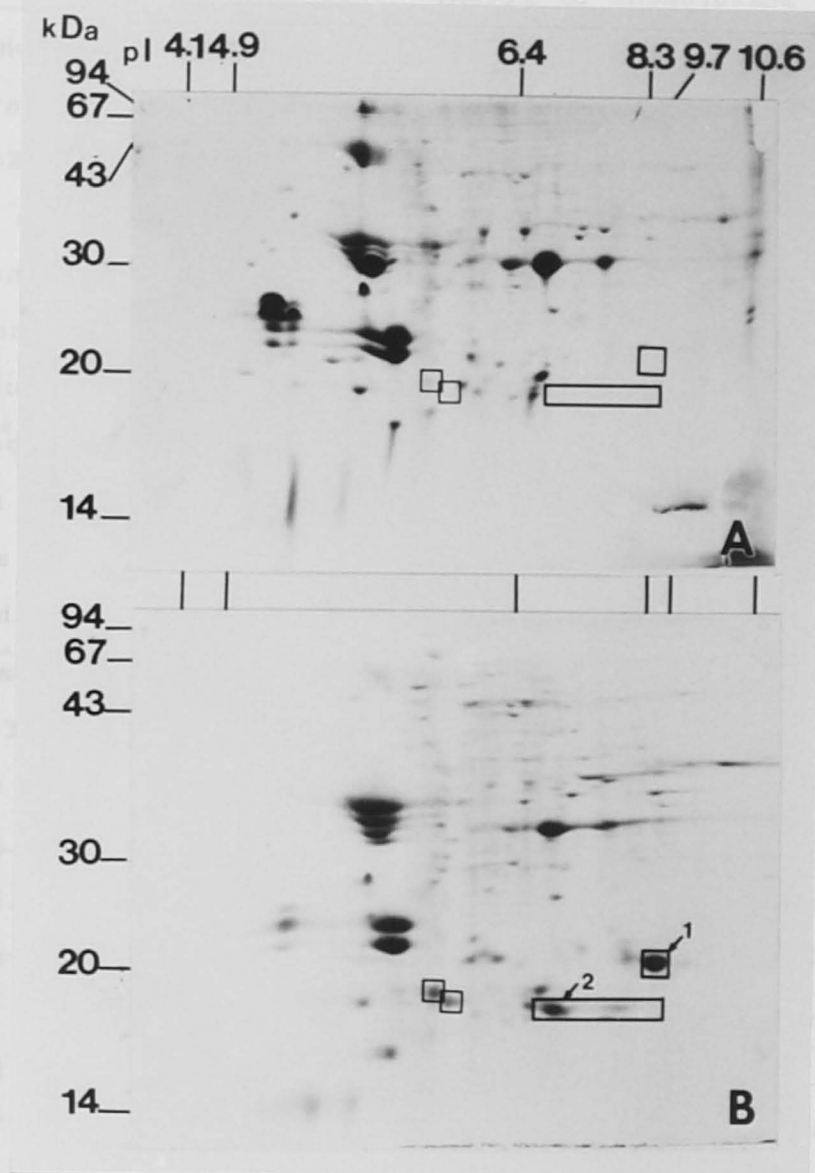


Fig. 2.2 A, B Two-dimensional polyacrylamide gel electrophoresis of the bark proteins of *Sophora japonica*.

A: harvested in June; B: harvested in December.

The polypeptide spots marked with square and rectangle are observed only in December (B). The same positions on the gel of June are also marked (A). The polypeptides indicated by arrows, 1, WP22 and 2, WP18 were purified. Antibodies against them were raised.

until the measurement by ELISA. WP22 was undetectable in July and WP18 was about 20% of the most abundant month, while the lectin retained about 50%. Total proteins in bark have been known to be more abundant in winter than in summer (Kozlowski 1979). Recently, it was reported that several protein species show such seasonal changes (Nsimba-Lubaki and Peumans 1986; Wetzel et al. 1989; Baba et al. 1990). Two roles are thought to be played by such proteins. One is storage of nitrogen during the dormant period (O'Kennedy and Titus 1979; Nsimba-Lubaki and Peumans 1986; Wetzel et al. 1989), and the other is the role for cold resistance in winter (Kang and Titus 1987). WP22 and WP18 would play either of the two, respectively. This is the first report for the measurement of the changes in the certain bark proteins other than lectin, and showing their profiles in relation to season. These profiles must be required to estimate their physiological roles in future. In present, these profiles suggested that the changes of WP22 might be intentionally fixed in relation to season, while that of WP18 might be a result of the counterplan to the environmental changes. According to this idea, the former can play for storage, and the latter for cold resistance.

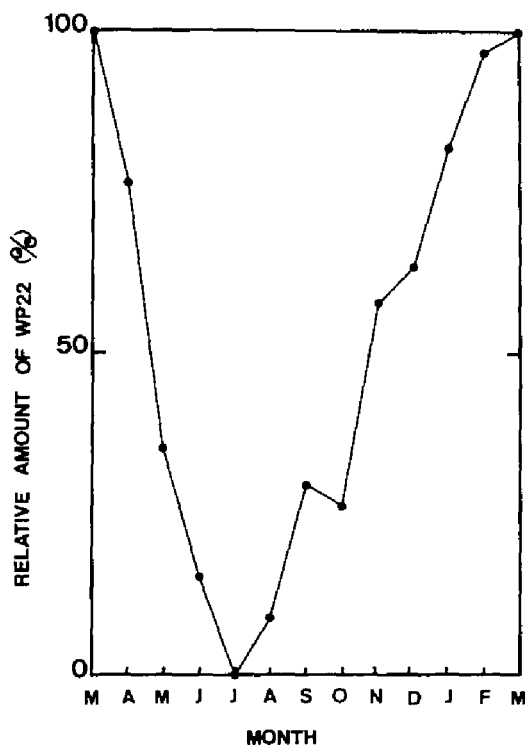


Fig 2.3 Seasonal changing profile of WP22 measured by enzyme linked immunosorbent assay.

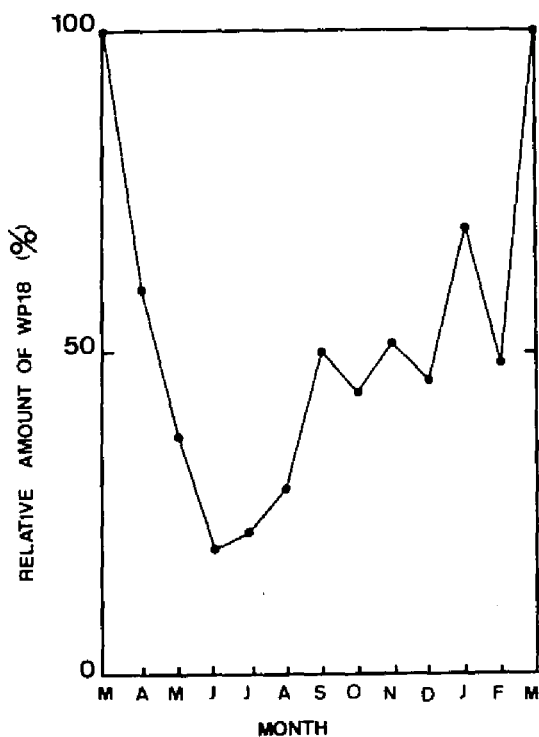


Fig. 2.4 Seasonal changing profile of WP18 measured by enzyme linked immunosorbent assay.

Conclusion

Lectin is the dominant protein in the inner bark of *S. japonica*, and its content in the bark changed during the year, the average amount in summer being about 50% of that in winter.

By immunohistochemistry the lectin was demonstrated to be localized in the ray and the axial parenchyma cells. Neither lectin nor other cross reactive materials were observed in the cambium, sieve tubes and companion cells. In xylem another lectin would be present (Baba et al. 1990a). The distribution and localization of the lectin changed in relation to the development of the bark tissue. The distribution of lectin in winter was similar throughout the inner bark. In contrast, in summer the innermost region hardly contained any lectin, and the outermost region contained less lectin than the middle. Lectin localization in tissues and cells differed also depending on tissue age. In new tissue, produced in the current year, lectin was absent in summer, located in the cytoplasmic layer between cell wall and vacuole in autumn, and sequestered in the vacuoles in winter. On the other hand, lectin in old tissue (formed in the previous year) was located mainly within the vacuoles throughout the year, with only very small contents in the cytoplasmic layer in autumn. Within the outermost (oldest) region, in which the lectin content was low in summer, the cells which bordered the outer bark never contained any lectin in summer.

The intracellular localization of the lectin in new tissue in autumn, determined by immuno-gold electron microscopy, was in the lumen of the endoplasmic reticulum (ER) and vesicles and hardly observed in the cytoplasmic matrix. From these findings it is concluded that lectin is synthesized on the ER most

vigorously in the new tissue in autumn, and that it is mainly consumed in the outermost bark regions, where dilatation occurs and/or where cork cambium is differentiated.

Further electron microscopical observations indicated that the bark lectin of *S. japonica* is localized in the Golgi apparatus. This result suggested that the lectin is transported through the Golgi apparatus on the way from the ER to the vacuole. On the other hand, it was sometimes observed that ER-like endomembrane contains electron-dense materials by conventional electron microscopy. Moreover, such endomembrane appeared to fuse to the tonoplast. Some of the lectin molecules or subunits may be transported through the Golgi apparatus and the others of them may be transported directly from ER to vacuole in the parenchyma cells of the bark of *S. japonica* (Baba et al. 1990b).

At least four other polypeptides, which were obviously abundant in winter and less in summer, coexisted with lectin in the bark of *S. japonica* were observed on the gels of SDS-PAGE and 2D PAGE. Two of such polypeptides, 18 kDa and 22 kDa in apparent molecular sizes, were measured on monthly basis by enzyme-linked immunosorbent assay using the antibodies raised against them. Both of them were most abundant in March, and decreased from spring to summer and increased from autumn to winter. The increasing patterns, however, were somewhat different between them. The 22 kDa polypeptide went linearly up, whereas the 18 kDa polypeptide went up with a fluctuation.

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